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Widely Distributed Mutations in the COL2A1 Gene Produce Achondrogenesis Type II/Hypochondrogenesis

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The COL2A1 gene was assayed for mutations in genomic DNA from 12 patients with achondrogenesis type II/hypochondrogenesis. The exons and flanking sequences of the 54 exons in the COL2A1 gene were amplified by a series of specific primers using PCR. The PCR products were scanned for mutations by conformation sensitive gel electrophoresis, and PCR products that generated heteroduplex bands were then sequenced. Mutations in the COL2A1 gene were found in all 12 patients. Ten of the mutations were single base substitutions that converted a codon for an obligate glycine to a codon for an amino acid with a bulkier side chain. One of the mutations was a change in a consensus RNA splice site. Another was an 18-base pair deletion of coding sequences. The results confirmed previous indications that conformation sensitive gel electrophoresis is highly sensitive for detection of mutations in large and complex genes. They also demonstrate that most, if not all, patients with achondrogenesis type II/hypochondrogenesis have mutations in the COL2A1 gene. Am. J. Med. Genet. 92:95–100, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: achondrogenesis; hypochondrogenesis; osteochondrodysplasia; COL2A1; collagen

INTRODUCTION

Type II collagen is the most abundant protein of the cartilage matrix and is essential for the normal embryonic development of the skeleton, for linear growth, and for the ability of cartilage to resist compressive forces [Piez, 1984; Prockop and Kivirikko, 1995]. Mutations in the gene for type II collagen, COL2A1, disrupt these processes and cause a variety of osteochondrodysplasias [Ritvaniemi et al., 1995; Kuivaniemi et al., 1997]. COL2A1 mutations were first identified in patients with spondyloepiphyseal dysplasia and hypochondrogenesis [Lee et al., 1989; Vissing et al., 1989]. Since then, over 40 mutations in the COL2A1 gene have been reported in patients with achondrogenesis type II/hypochondrogenesis (ACG2/HCG), spondyloepimetaphyseal dysplasia Strudwick type, Kniest dysplasia, the Stickler and Wagner syndromes, and mild osteochondrodysplasia phenotypes with early-onset osteoarthrosis as a major manifestation [Kuivaniemi et al., 1997; Spranger et al., 1994]. Most of the mutations were single base changes that each caused a single amino acid substitution for one of the obligate glycine residues that appear in the repeating Gly-X-Y triplet characteristic of collagen α-chains. However, RNA splicing mutations, partial gene deletions, insertions, and duplications were also observed. In addition, five families, three or whom perhaps were related through an early ancestor, had a single base mutation that changed the codon for arginine at residue 519 to a cysteine codon (R519C) and produced primary generalized osteoarthrosis with or without evidence of a mild osteochondrodysplasia phenotype [Ala-Kokko et al., 1990; Pun et al., 1994; Williams et al., 1995; Bleasel et al., 1995; Holderbaum et al., 1996]. In three unrelated

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TABLE I. Clinical Data on Patients

ISDR[a] number	Gestation age (weeks)	Gender	Radiograph diagnosis	Cartilage morphology	Hydrops	Midface hypoplasia	Cleft palate	Other abnormalities
85-155	35	F	ACG2	Hypervascular, hypercellular	+	+	+	
87-051	23	M	ACG2	Sparse collagen fibrils, hypervascular, large foamy lacunae	+	+	—	Polyhydramnios
89-123	30	F	ACG2	Sparse matrix, hypervascular, chondrocyte RER inclusions	+	+	+	Polyhydramnios
89-131	22	F	ACG2	Sparse matrix, hypervascular, chondrocyte RER inclusions	+	+	Unknown	
89-200	36	F	ACG2	Hypervascular, chondrocyte RER inclusions	+	+	+	Polyhydramnios
91-215	24	M	ACG2	Sparse matrix, hypervascular, chondrocyte RER inclusions	+	+	Unknown	Polyhydramnios
92-197	21	M	ACG2/HCG	Sparse matrix, hypervascular	+	Unknown	Unknown	
92-240	15	F	ACG2	Hypervascular, hypercellular cartilage	+	+	+	Cystic hygroma
93-078	23	F	ACG2	Unknown	–	+	–	Polyhydramnios
93-260	28	F	ACG2	Foamy lacunae	+	+	+	Polyhydramnios, dysplastic left kidney, bilateral equinovarus
94-160	17	F	ACG2	Hypervascular, hypercellular	+	+	Unknown	Bilateral equinovarus
95-085	32	F	ACG2	Sparse matrix, hypercellular, foamy lacunae, chondrocyte RER inclusions, disorganized growth plate	+	+	Unknown	Polyhydramnios, cystic hygroma, hydrocephalus

[a]International Skeletal Dysplasia Registry.

patients, a R75C substitution produced a similar phenotype [Bleasel et al., 1995; Williams et al. 1993]. In contrast, a R789C substitution, identified in two unrelated patients, resulted in spondyloepiphyseal dysplasia [Chan et al., 1990, 1993].

Although a large number of mutations in the COL2A1 gene have been reported in patients with varying phenotypes, it has been difficult to define the true incidence of such mutations among patients with a specific phenotype [Ritvaniemi et al., 1995]. This is primarily because patient cartilage is usually not available for analysis at the protein or mRNA level, and the COL2A1 gene has a complex structure composed of 54 exons. Thus complete examination of the COL2A1 gene for mutations is technically difficult. The highest number of mutations reported in the COL2A1 gene was in patients with the perinatally lethal phenotypes of ACG2 and HCG, with 13 reported mutations [Ritvaniemi et al., 1995; Kuivaniemi et al., 1997; Mundlos et al., 1996]. To determine the sensitivity of the relatively simple technique [Ganguly et al., 1993] of conformation sensitive gel electrophoresis (CSGE) for detecting COL2A1 mutations, and to examine the spectrum of mutations that can produce ACG2/HCG, we carried out mutation analysis for 12 patients. Mutations were identified in all 12 patients, demonstrating that CSGE is highly sensitive for detecting mutations in the COL2A1 gene, that ACG2/HCG appears to be produced exclusively by COL2A1 mutations, and that the mutations are distributed across the gene more widely than previously assumed.

MATERIALS AND METHODS

Clinical Ascertainment

Twelve fetuses with ACG2 or HCG were ascertained through the International Skeletal Dysplasia Registry (ISDR). The gestational ages of the fetuses ranged from 15 to 35 weeks. Clinically the fetuses presented with findings typical for the ACG2/HCG spectrum: hydrops, midface hypoplasia, cleft palate, severe shortness of the rhizo- and meso-melic portions of the limbs, and

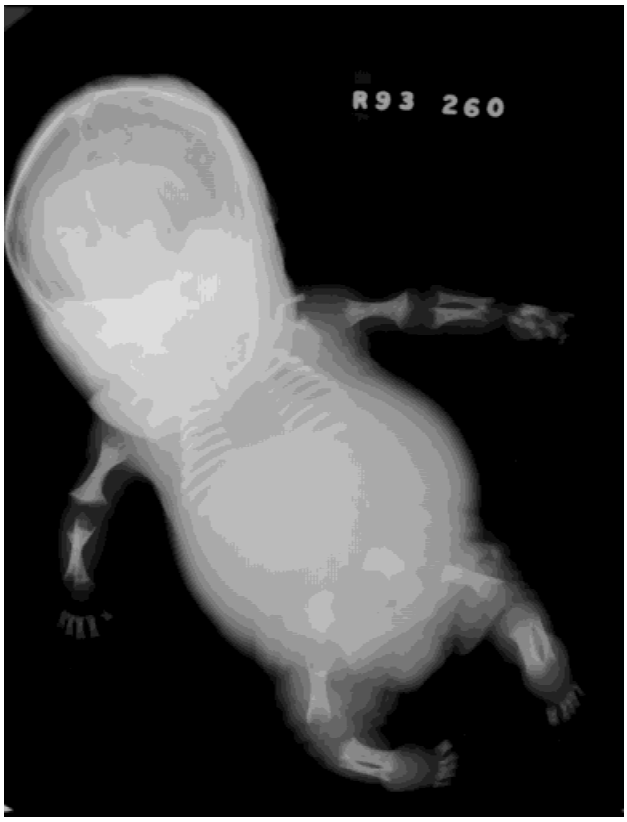

Fig. 1. AP view of fetus R93-260.

equinovarus (summarized in Table I) [Chen et al., 1981]. The radiographs were also consistent with the diagnosis, demonstrating severely underossified vertebral bodies, small iliac bones, shortened tubular bones with metaphyseal cupping, lateral bowing of the femurs, and unossified pubic and ischial bones (Fig. 1).

Cartilage morphology was examined in 11 of the cases (see Table I) by published methods [Brodie et al., 1998]. In all cases, the characteristic findings of hypervascular/hypercellular cartilage, foamy lacunae, a sparse matrix, and rough endoplasmic reticulum inclusions in chondrocytes (Fig. 2) were also consistent with the diagnosis of ACG2/HCG [Horton et al., 1987].

Mutation Analysis

Genomic DNA was extracted from blood samples or from cultured skin fibroblasts. The exons and the flanking sequences of the 54 exons of the COL2A1 gene were amplified by a series of specific primers (http://www.mcphu.edu/medschool/centers/matrix/images/col2a1pr.htm) based on the published gene sequence [Ritvaniemi et al., 1995; Williams et al., 1993, 1995; Ala-Kokko and Prockop, 1990]. Genomic DNA was amplified in a 40 μl volume by thermal cycling at 95°C for 10 min for one cycle, followed by 95°C for 40 sec, 60°C for 40 sec, and 72°C for 50 sec, for 35 cycles. This was

Fig. 2. Transmission electron micrograph of cartilage from fetus R95-085. Final magnification: 20,000×.

followed by heteroduplex formation steps: 95°C for 5 min and 68°C for 30 min. CSGE analysis was performed as described elsewhere [Ganguly et al., 1993, Körkkö et al., 1998a,b]. The gels were examined with a hand-held UV illuminator (short wave) to identify regions containing homoduplex and heteroduplex fragments. Appropriate regions were excised from the gels, transferred to filter paper, and analyzed further in a UV-image analyzer with a CCD camera (DOC-IT™ Gel Documentation System; UVP). The image from the monitor was recorded by a thermal printer. After treatment with exonuclease I and shrimp alkaline phosphatase, the sequences of PCR products containing heteroduplexes were determined by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer, Foster City, CA) and an automated instrument (ABI PRISM 377 Sequencer; Perkin-Elmer). PCR products that contained deletions in one allele were purified from agarose (QIA II Gel Extraction Kit; Qiagen, Chatsworth, CA) and cloned into a plasmid (pT7 Blue T-Vector Kit; Novagen, Madison, WI) prior to sequence analysis.

RESULTS

All 54 exons and the flanking sequences of the COL2A1 gene were amplified by PCR from all 12 patients with ACG2/HCG. The samples were then analyzed for mutations in the COL2A1 gene. Mutations were first identified by unique heteroduplexes detected by CSGE (Fig. 3), and sequence analysis of the PCR products was used to define the specific mutations. The analysis identified novel disease-causing mutations in all patients (Table II). Ten of the mutations were substitutions for obligate glycines in the triple helical domain of the type II collagen molecule, one was a deletion of 18 bp of coding sequence, and one was predicted to cause exon skipping. In addition to the disease-causing mutations, over 30 neutral polymorphisms were detected (Table III).

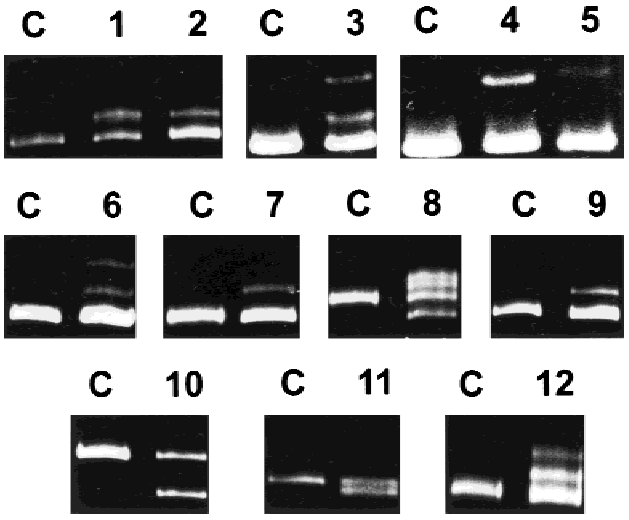

Fig. 3. The CSGE patterns caused by the mutations. Numbers indicate the patient numbers. C, Control. Lanes 1–12 correspond to patients ISDG 89–131 to 94–160 in Table II.

TABLE II. Mutations in COL2A1 Causing Achondrogenesis II/Hypochondrogenesis

Patient		Mutation in COL2A1	
ISDG number	Diagnosis	Exon	Mutation
89-131	ACG2	19	G253V
89-200	ACG2	19	G253D
85-155	HCG	32	IVS34 + 1G > A
92-240	ACG2	33	G571D
87-051	ACG2	33	G580R
91-215	ACG2	34	G595R
92-197	ACG2/HCG	39	G694E
95-085	ACG2	40	G748D
89-123	ACG2	41	G781S
93-078	HCG	44	G817-V822del6aa
93-260	ACG2	44	G865V
94-160	ACG2	46	G919R

DISCUSSION

On phenotypic, radiographic, and morphologic grounds [Chen et al., 1981; Horton et al., 1987], as well as in the light of mutation analyses of the COL2A1 gene, it was previously assumed that achondrogenesis type II/hypochondrogenesis was caused exclusively by COL2A1 mutations. However, because of limitations in the sensitivity of mutation detection methods employed, no systematic analysis of patients was possible. Furthermore, for collagen genes, including COL2A1, the large number of highly homologous exons has presented a formidable technical barrier for the complete detection of mutations [Prockop and Kivirikko, 1995].

TABLE III. Polymorphisms Detected in the COL2A1 Gene

Fragment for exon number	Change
1	TCG-ACG (SerN9-Thr)
1	IVS1+18g/c
5a	GAA-GAT (GluN142)
5b	GGC-GGA (GlyN168)
6	IVS5-27t/a
7	IVS6-7g/a
7	IVS7+15g/t
9	IVS9+15g/a
9	IVS9+42delg
10	IVS9-42g/a
18	IVS18+7g/a
20	IVS19-11c/t
30	IVS29-49g/t
30	IVS30+7a/g
30	IVS30+12g/c
32	IVS31-53^-52insg
32	GGC-GGT (Gly565)
32	IVS32+73c/t
33	IVS32-32t/c
33	IVS32-22g/a
33	IVS33+68g/a
34	IVS33-56g/t
34	AAC-AAT (Asn600)
34	IVS34+84c/g
36	IVS35-74a/c
40	IVS39-45delg
50	GTT-ATT (Val1131Ile)
50	IVS50+48-+49delct
51	IVS50-14c/g
52	3′UTR+4c/t

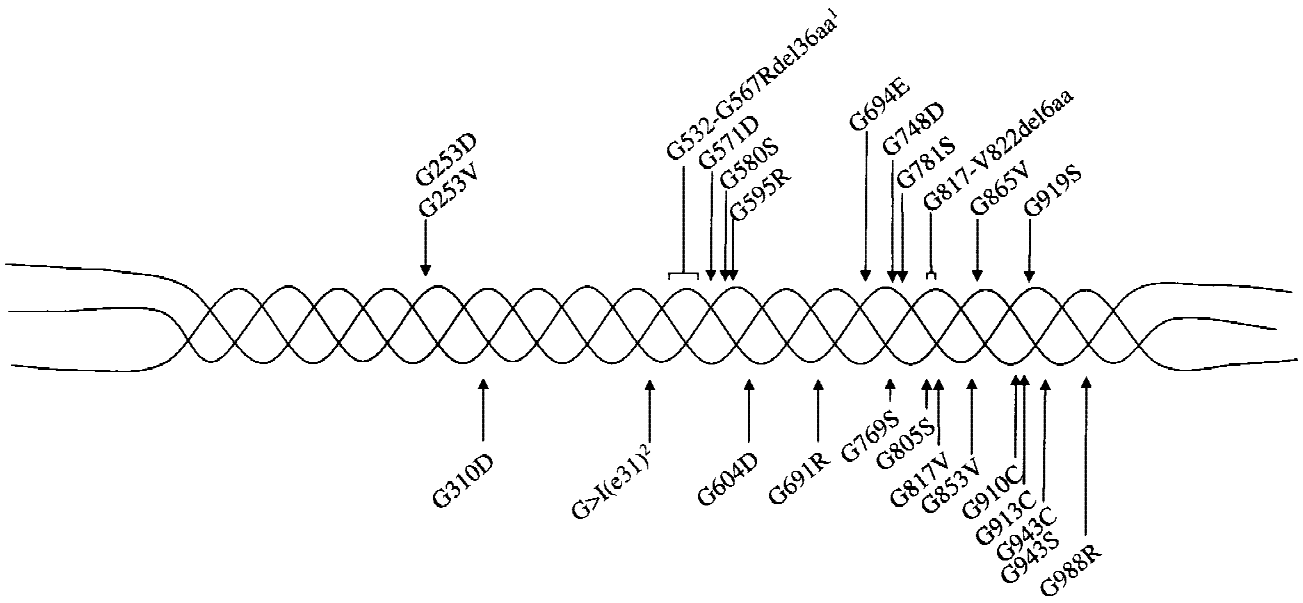

Fig. 4. Schematic of the type II collagen molecule indicating sites of mutation causing achondrogenesis type II/ hypochondrogenesis. Arrows above molecule: the sites of mutations reported here. Arrows below molecule: the sites of previously reported mutations. [1]The mutation detected was IVS34+1G→A; the figure presents the predicted effect on the amino acid level. [2]The exact amino acid number not known [Mundlos et al., 1996].

The use of CSGE [Ganguly et al., 1993, 1997; Gatalica et al., 1997; Körkkö et al. 1998a,b; Philippe et al., 1997] has greatly facilitated the analysis of such genes because it has provided a relatively simple means of scanning PCR products for single base mismatches or other allelic differences. The procedure has been shown to be sensitive enough to detect essentially all single base changes in several hundred alleles of the COL1A1, COL1A2, and COL2A1 genes [Ganguly et al., 1993; Körkkö et al., 1998b]. The results presented here further demonstrate the sensitivity of this analytic method and suggest that the phenotypes studied are caused primarily, if not exclusively, by defects in type II collagen.

All 13 previously reported mutations in the ACG2/HCG patients caused conversion of an obligate glycine to a bulkier amino acid [Kuivaniemi et al., 1997; Mundlos et al., 1996]. The data presented here demonstrate that in-frame deletions and RNA splicing mutations can also produce phenotypes at the lethal end of the type II collagenopathy spectrum. Based on a similar hypothesis that emerged from analysis of the type I collagen genes in patients with osteogenesis imperfecta [Byers, 1993], it was suggested that type II collagen gene mutations in patients with more severe phenotypes might cluster at the carboxyl-terminal end of the triple helical domain [Murray et al., 1989]. This hypothesis was consistent with mutations previously identified in ACG2/HCG since, with the exception of a G310D substitution characterized in a case of ACG2 [Bonaventure et al., 1995], all mutations were located in the region of the gene corresponding to the carboxyl-terminal half of the triple helical domain. Mutations in the cohort studied here demonstrate that lethal COL2A1 mutations are more widely distributed in the molecule, a result not consistent with the gradient hypothesis (Fig. 4).

Studies with synthetic peptides [Chan et al., 1997], as well as genotype/phenotype comparisons in patients [see Westerhausen et al., 1990; Wang et al., 1993; Marini et al., 1993], demonstrate that the sequence context and the specific amino acid substitutions for glycine influence the functional consequences. However, it has been difficult to establish firm relationship between the mutation and the severity of the phenotype because there are several examples of the same substitution of an amino acid for glycine at the same position that produce phenotypes that vary from mild or moderate to lethal (Kuivaniemi et al., 1997). The phenotypic variability seen with similar or identical mutations in collagen genes is consistent with the phenotype variability recently seen in monozygotic twins with identical mutations in a variety of genes [see Hall, 1996; Wyszyski et al., 1996; Yamagishi et al., 1998].

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